Ion channels on synaptic vesicle membranes studied by planar lipid bilayer method

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ABSTRACT An anion selective channel and three types of cation selective channels were found in planar lipid bilayers incorporating synaptic vesicles from rat brains. In asymmetric KCl solutions (*cis:* 300 mM/*trans:* 150 mM), the anion selective channel showed a single-channel conductance of 94 pS and was inactivated by negative voltages and by 4-acetoamido-4′-isothiocyanostilbene-2,2′-disulfonic acid disodium salt (SITS). In the same solution, single-channel conductances of three types of cation selective channels were 250 pS (Type 1), 248 pS (Type 2), and 213 pS (Type 3), respectively. These channels resembled one another in single-channel conductances but were different in gating behaviors. Type 1 channel, which was most frequently observed, had a remarkable subconducting state (175 pS). Type 2 channel had a flickering state that increased as the potential became more positive, and a long inactive state that increased as the potentials were more negative. Type 3 channel, which was also sensitive to the potentials, had the open-channel probability increased as the potential became more positive.

INTRODUCTION

It is believed that the nerve transmission occurs as follows (1). When depolarization is conducted to the nerve terminal, voltage-dependent Ca^{2+} channels at the active zones are opened, and extracellular Ca^{2+} ions enter the nerve terminal. The increased intracellular Ca^{2+} triggers the release of neurotransmitters into the synaptic cleft and thus signals are transmitted to the adjacent neurons through the postsynaptic receptors. The release mechanism of neurotransmitters is generally accepted to be exocytosis (2): neurotransmitters, which are concentrated in synaptic vesicles, are released into the synaptic cleft as the result of the membrane fusion of synaptic vesicles to the presynaptic plasma membranes.

It has been reported that the uptakes of neurotransmitters such as monoamine (3), glutamate (3–7, 9), and γ-aminobutyric acid (3, 8, 9), into synaptic vesicles are mediated by the electrochemical gradient of protons across the synaptic vesicle membrane generated by a vacuolar-type H^+-ATPase. The proton electrochemical gradient involves the pH gradient and the membrane potential across the synaptic vesicle membrane, and recently it was reported that the ratio of the pH gradient and the membrane potential depend on the Cl^- concentration outside synaptic vesicles (3, 6–8). On the other hand, in mast cells, the studies of exocytosis measuring the capacitance and the conductance of cell membranes by using the whole-cell patch-clamp method has been suggested by the formation of channel-like structures, "fusion pore," at the early step in exocytosis (10–13). Considering these findings, it is important to characterize ion channels existing on synaptic vesicle membranes in order to understand the precise mechanism of nerve transmission.

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In this paper, we investigated ion channels on synaptic vesicle membranes from rat cerebral cortex by the planar lipid bilayer method and found new types of ion channels: one anion selective channel and three cation selective channels that are different from each other in gating behaviors.

MATERIALS AND METHODS

Preparation of synaptic vesicles

Synaptic vesicles were prepared from cerebral cortex of Sprague-Dawley rats by the methods of Huttner et al. (14), except that the gel filtration step was omitted. Brain homogenate was centrifuged at 800 *g* for 10 min, and the supernatant was centrifuged at 9,200 *g* for 15 min. The pellet was suspended in sucrose buffer (0.32 M sucrose, 4 mM Hepes-NaOH, 1 mM EGTA, pH 7.3), and the suspension was centrifuged at 10,200 *g* for 15 min. The pellet (crude synaptosomal fraction) was lysed with hypotonic buffer (8 mM Hepes-NaOH, pH 7.4). The lysate was centrifuged at 25,000 *g* for 20 min, and the supernatant was centrifuged at 165,000 *g* for 2 h. The pellet was suspended in 40 mM sucrose buffer, and placed on continuous sucrose density gradient (50–800 mM). After centrifugation at 65,000 *g* for 5 h, a broad band at 200–400 mM sucrose was collected as the synaptic vesicle fraction.

Planar bilayer system

Planar bilayers were formed from a 2:1 mixture of phosphatidylethanolamine (PE) and phosphatidylserine (PS) (20 mg/ml in *n*-decane) as described previously (15), and synaptic vesicles were fused into the membranes. The electrical conductance of the membranes was measured by a current-to-voltage converter under voltage-clamp conditions and was monitored with a pen-recorder (VP6523A; Panasonic Co., Osaka), and simultaneously recorded with a videotape recorder (BR2400; JVC Co., Yokohama) after digitization with a digital audio processor (PCM-501ES; Sony Corp., Tokyo) modified for dc recording.

The solutions used were composed of 300 mM KCl in the *cis* side (the side to which synaptic vesicles were added) and 150 mM KCl in the *trans* side (the opposite side). Both solutions contained 4 mM Hepes-NaOH, 2 mM $CaCl_2$, and 1 mM EGTA adjusted to pH 7.3. The voltage across the membrane was defined with respect to the *trans* side. Experiments were carried out at room temperature (20–25°C).

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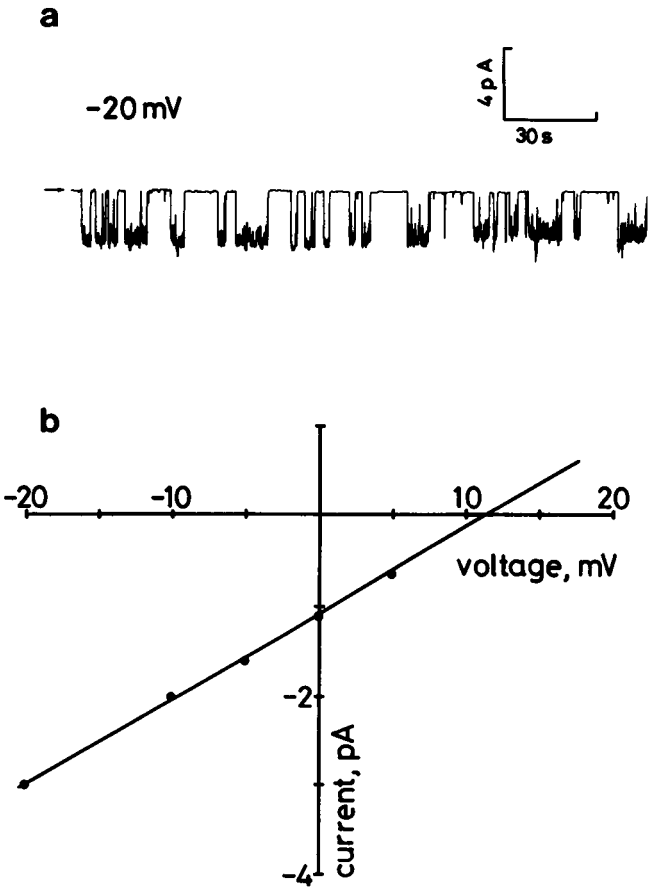

FIGURE 1 (*a*) Single-channel current fluctuations of an anion selective channel. Single-channel current fluctuations were recorded with asymmetrical solutions (*cis*, 300 mM KCl/*trans*, 150 mM KCl). The arrow indicates closed level. Channel opening is downward. Holding potential was −20 mV. (*b*) I–V relationship of single-channel currents. The single channel conductance was determined as 94 pS from the slope.

Chemicals

Bovine brain phosphatidylserine and egg yolk phosphatidylethanolamine were purchased from Sigma Chemical Co., St. Louis, MO). 4-acetoamido-4′-isothiocyanostilbene-2,2′-disulfonic acid disodium salt (SITS) was obtained from Nakarai Chemicals, Kyoto, Japan. Other reagents were commercial products of analytical grade.

RESULTS

Characteristics of the anion selective channel

Fig. 1 *a* shows a single-channel current fluctuation of an anion channel incorporated into a planar bilayer, and the single-channel currents were plotted in Fig. 1 *b* as a function of holding potentials. This type of anion channel was observed 3 times out of a set of 43 incorporations of ion channels into lipid planar bilayers. The single-channel conductance was determined as 94 pS from the slope. The reversal potential was ~12 mV. Since the contribution of the junctional potential is less than 1 mV in our experimental system, the difference from the ideal anion selective channel (17.6 mV) is attributable to the low selectivity of the channel. The calculated selectivity ratio, P_{Cl}/P_k, was ~6. In most cases, several channels were simultaneously incorporated into a planar bilayer. Since the channel trace derived from 9 to 10 channels incorporated into the planar bilayer showed random fluctuations entirely, each channel seems to open and close independently. But we could not obtain the quantitative relationship between the open-channel probabilities at the single-channel level and those at macroscopic level. This type of channel was inactivated at positive voltages and was relatively voltage independent at negative voltages.

Fig. 2 shows the effect of SITS on the anion channel. SITS, a stilbene derivative, is known as an anion channel blocker (16). After the addition of 50 μM SITS to the *cis* side, the open-channel probability decreased without

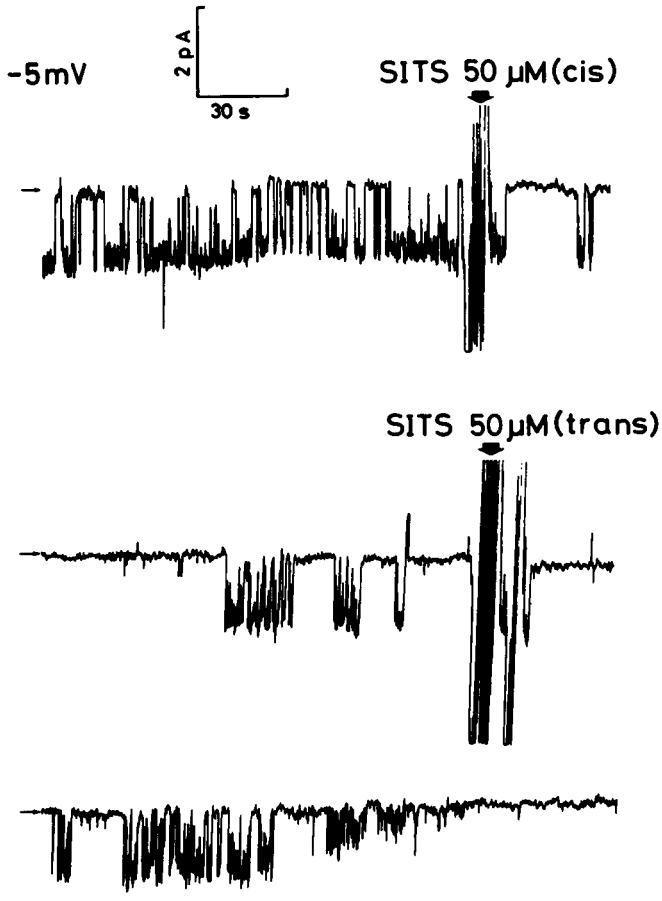

FIGURE 2 Effect of SITS on an anion selective channel. An addition of 50 μM SITS to the *cis* side causes the decrease of the open-channel probability, and an additional SITS to the *trans* side causes the gradual decrease of the single channel conductance, and finally blocks the channel. The arrows indicate closed level. Channel opening is downward. Solutions were the same as in Fig. 1. Holding potential was −5 mV.

changing the single-channel conductance. An additional SITS to the *trans* side caused the gradual decrease of the single-channel conductance, and completely blocked the channel within 10 min. White and Miller (16) reported that the effect of SITS on Cl^- channels taken from the *Torpedo* electric organ was rapid, and a relatively high concentration was required for inhibition of channels (K_i = 120 μM). Additionally, SITS was effective only from the *cis* side. Compared with this, Cl^- channels in this paper were inhibited by SITS at relatively low concentrations within several minutes after the addition. Further, 50 μM SITS in the *cis* side decreased the open-channel probability and an additional 50 μM SITS to the *trans* side closed the channel completely, suggesting that the binding sites for SITS exist on both sides.

Characteristics of three types of cation selective channels

In addition to the anion channel, three types of cation selective channels (termed Type 1, Type 2, and Type 3) were observed. The numbers of occurrences of these cation channels which we could distinctly observe at single-channel level were 8, 3, and 4 out of a set of 43 incorporations of ion channels into lipid planar bilayers, respectively. These resemble each other in the single-channel conductances, but are different in their gating behaviors. Fig. 3 shows a single-channel current fluctuation of Type 1 channel (*a*) and the single-channel current–voltage relationship (*b*). The single-channel conductance was determined as 250 pS from the slope. The reversal potential was about −10 mV, indicating low selectivity such as P_K/P_{Cl} = 4. As shown in Fig. 3 *a*, Type 1 channel has a remarkable subconducting state. The I–V relationship of the subconducting states is illustrated by the dotted line in Fig. 3 *b*. The change in the currents was linear with applied voltages, and the substate conductance was determined as 175 pS from the slope. Type 1 channel was most frequently observed when compared with other cation channels, and in most cases two or three channels were incorporated into the planar bilayer at the same time. Type 1 channel did not show remarkable changes in conductance, even at negative potentials, but fluctuation became far from discrete, and thus gating behavior seems to change with potentials. When decamethonium (1.4 mM) was added to the *cis* side, the single-channel conductance decreased slightly to 85% at 10 mV. High concentration of decamethonium was required for blockade. Further analysis was not done.

Fig. 4 shows a single-channel current fluctuation of Type 2 channel and the single-channel current–voltage relationship. The single-channel conductance was determined as 248 pS from the slope, and the reversal potential was about −12 mV, corresponding to P_K/P_{Cl} = 6. Type 2 channel has a series of flickering states, which increase as the voltage becomes more positive, and a relatively long inactive state, which increases as the voltage becomes more negative.

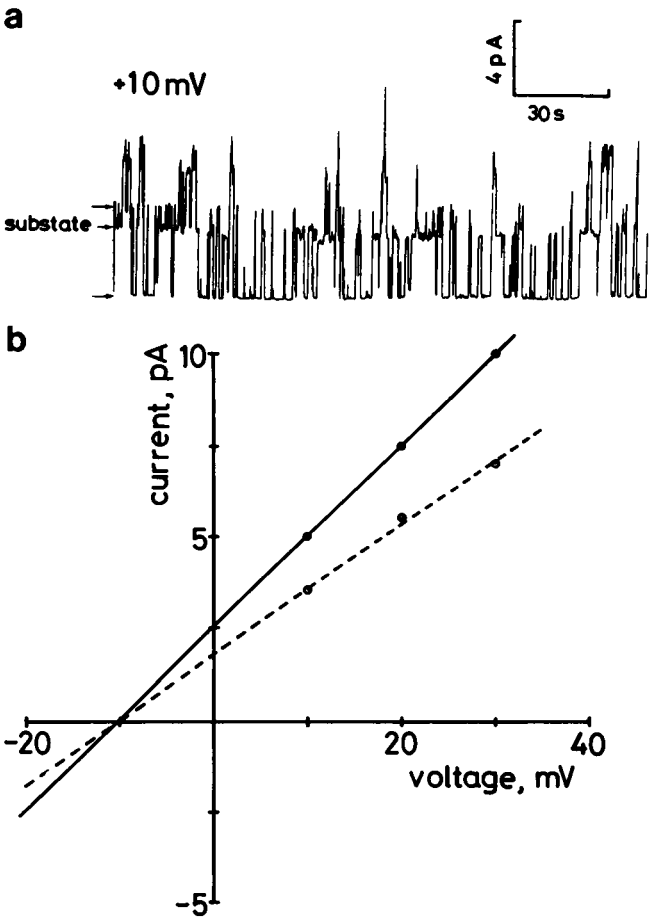

FIGURE 3 (*a*) Single-channel current fluctuations of Type 1 cation channel. The arrows consecutively indicate closed level, subconducting level, and full-opening level from the bottom. Channel opening is upward. Solutions were the same as in Fig. 1. Holding potential was +10 mV. (*b*) I–V relationships of single channel currents and subconducting currents. The solid line indicates the I–V relationship of the full-opening state and the dotted line, subconducting state. The conductances were determined as 250 pS and 175 pS from the slope, respectively.

Fig. 5 shows a single-channel current fluctuation of Type 3 channel and the single-channel current–voltage relationship. The single-channel conductance was determined as 213 pS from the slope. Since the reversal potential was about −17 mV, the channel is more ideal as a cation selective channel than Type 1 and Type 2. Type 3 channel has a remarkable voltage dependency; the open-channel probability increases as the voltage becomes more positive. This channel was more sensitive to decamethonium than Type 1 channel. An addition of 300 μM decamethonium to the *cis* side decreased the single-channel conductance to 85% at 10 mV. Further analysis was not done.

As far as the effects of TEA on these cation channels are concerned, we could not get results at the single-

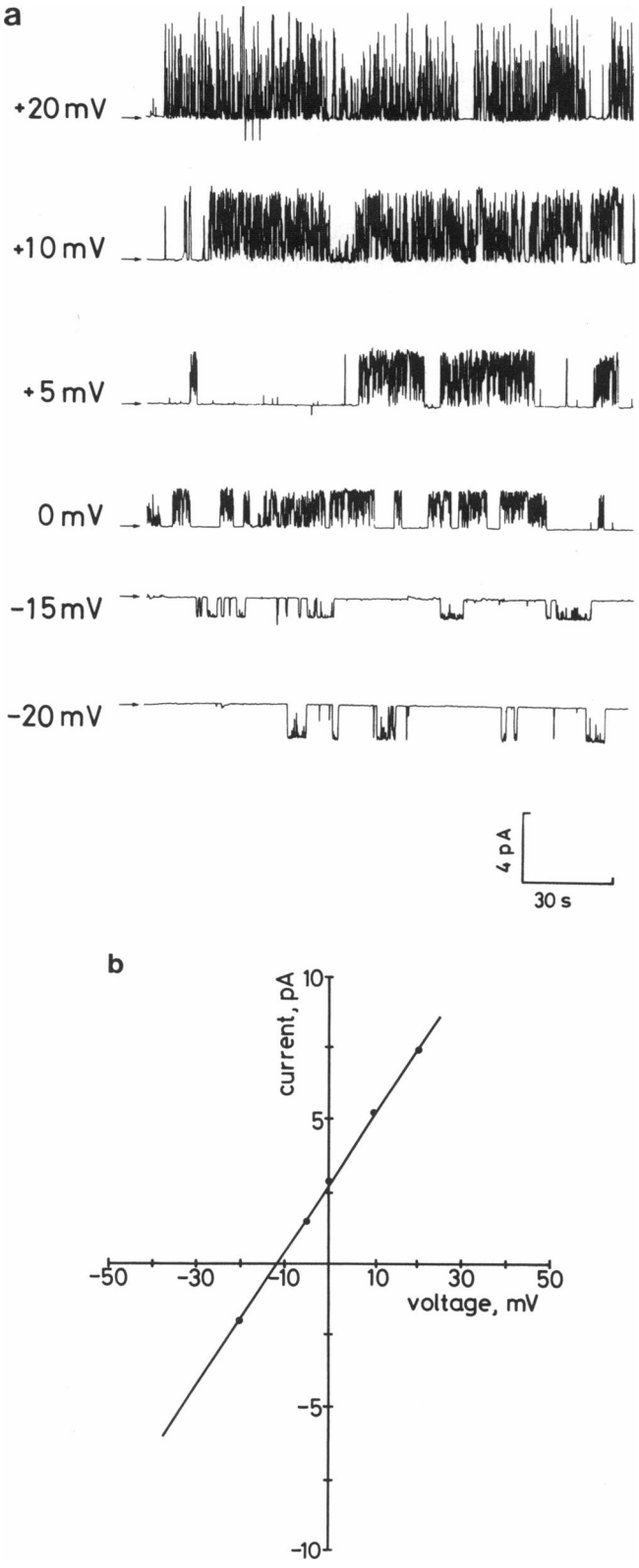

FIGURE 4 (*a*) Single-channel current fluctuations of Type 2 cation channel at various potentials. A series of flickering state increases as the voltage becomes more positive, and the relatively long inactive state increases as the voltage becomes more negative. The arrows indicate closed level. Channel opening is upward for positive voltages and downward for negative voltages. Solutions were the same as in Fig. 1. (*b*) I–V relationship of single-channel currents. The single-channel conductance was determined as 248 pS from the slope.

channel level. However, it was observed that an addition of 10 mM TEA to the *trans* side decreased macroscopic cation current to 25%. Although we could not discriminate which types of channel were blocked by TEA, the result suggests the existence of TEA-sensitive cation channels in our preparation. Further, an examination of the effect of Ca^{2+} on these ion channels is interesting and important for understanding their function. However, we could not get any effect of Ca^{2+} (up to mM) for all channels at present.

DISCUSSION

The experiments in this paper indicate that there are several types of ion channels on synaptic vesicle membranes. At present, the physiological roles of these channels for synaptic functions are not clearly understood, but so far there have been several reports that suggest the existence of ion channels in synaptic vesicles (3, 6–8) and secretory granules (10–13). For example, many investigators have indicated that the various transmitters, such as glutamic acid (3–7, 9) GABA (3, 8, 9), monoamine (3), and glycine (8), were taken up into synaptic vesicles by energy-dependent transport systems, and recently it has been shown that these uptakes, which were mediated by a proton electrochemical gradient across the synaptic vesicle membranes generated by a vacuolar-type H^+-ATPase, were dependent on the concentration of Cl^- as a permeant anion outside synaptic vesicles (6–8). This suggests the presence of Cl^- transporters on synaptic vesicle membranes, but the molecular entity is not clear. Further, electrophysiological studies concerning exocytosis in mast cells suggested the formation of channel-like structures, "fusion pores," at the early step in exocytosis, so that ion channels were thought to be important for exocytosis (10–13).

Therefore, to elucidate the mechanism for transmitter release, we think that characterization of ion channels on synaptic vesicle membranes is important as the first step of the study. Rahamimoff and co-workers (17) found K^+ selective channels in cholinergic synaptic vesicles purified from electric organs of *Torpedo marmorata* and Thomas and co-workers (18) reported that synaptophysin, a major membrane protein of synaptic vesicles, possesses the channel-forming activity. However, no other report has been found regarding ion channels in synaptic vesicles studied by electrophysiological methods.

In this paper, we found that the synaptic vesicles possess one type of anion channel and three types of cation channels. The channels presented here seem to be different from those reported by Rahamimoff (82, 102.5, and 205.7 pS, reference 17) and Thomas (150 pS, reference

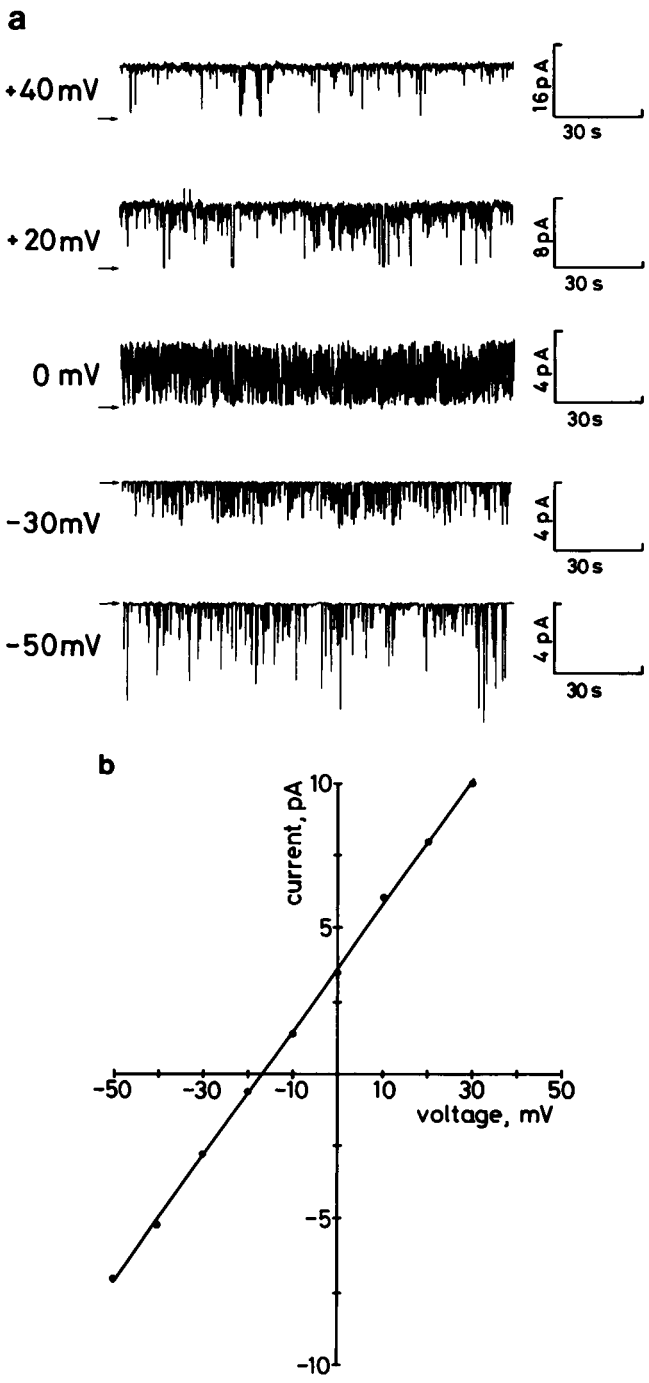

FIGURE 5 (*a*) Single-channel current fluctuations of Type 3 cation channel at various potentials. The open channel probability increases as the voltage becomes more positive. The arrows indicate closed level. Channel opening is upward for positive voltages and downward for negative voltages. Solutions were the same as in Fig. 1. (*b*) I–V relationship of single channel currents. The conductance was determined as 213 pS from the slope.

18) in many points, especially in the conductance. The synaptic vesicle fraction used in our studies shows a high degree of purity as indicated through electron microscopy (Sato, M., M. Kasai, and H. Ishikawa, unpublished observation). However, the possibility of contamination of a small amount of presynaptic plasma membranes in the fraction vesicles cannot be ruled out. Thus, there have been a few reports about ion channels on presynaptic plasma membranes. Among them are the cation channels, presented by Nomura et al. (19) and Farley et al. (20), which resemble the Type 3 channel discussed in this paper in conductance and gating behavior involving voltage dependency. Further, the anion channel termed Type II channel by Nomura et al. (21) is also similar to the anion selective channel in this paper. Since transmitters are generally considered to be released by exocytosis accompanied by the membrane fusion of synaptic vesicles with presynaptic plasma membranes, it is likely that the same kinds of channels are found on both synaptic vesicle membranes and presynaptic plasma membranes. In the present experiments, the cation selective Type 2 and Type 3 channels and the anion selective channel were rarely observed, so that these three types of channels may have originated from the contaminations of the presynaptic plasma membranes.

Different types of cation channels were seldom incorporated into the planar bilayers at the same time. This may indicate that the types of ion channels vary with those of synaptic vesicles. Maycox et al. (22) proposed that the ratio of the pH gradient and the membrane potential across the synaptic vesicle membranes varies with the Cl^- concentration outside the synaptic vesicles, and this causes the difference in the kinds of transmitters taken up into the synaptic vesicles. The different kinds of transmitters stored in the synaptic vesicles may possibly be regulated by different kinds of ion channels on their membranes.

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